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Rapid solution assays for retroviral integration reactions and their use in kinetic analyses of wild-type and mutant Rous sarcoma virus integrases

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ABSTRACT **A rapid method for quantitating products of the oligodeoxynucleotide processing reaction *in vitro* has been developed to facilitate enzymatic studies of the retroviral integrases. Unlike earlier procedures, this assay does not depend on polyacrylamide gel electrophoresis but separates products by batch adsorption to PEI-cellulose. A joining assay has also been modified, to facilitate measurement of the two distinct steps in the integration reaction under parallel conditions. Since these methods allow quantitation of numerous samples in a short period of time, they are especially useful for investigation of kinetic parameters and to measure the effects of possible inhibitors of integrase. These assay systems were used to examine the enzymatic activity of wild-type Rous sarcoma virus integrase and selected mutant proteins with substitutions of single conserved amino acids. In contrast to previous studies, reactions were performed under conditions of substrate excess, and rates, rather than yields of product generated after a given period of incubation, were determined. The results showed that substitutions of several highly conserved residues in what is most likely an evolutionarily conserved catalytic domain of the integrases resulted in a 4- to 10-fold decrease in the apparent rate of processing relative to wild type, under optimized standard conditions. Changing an invariant acidic residue reduced the rate by ≈60-fold. When joining activity was determined, the relative effects of the substitutions tested generally paralleled the results with processing. However, with both wild-type and mutant integrase proteins, the linear phase of the joining reaction was preceded by what appears to be an exponential "burst" phase.**

After a retrovirus infects a cell, the reverse-transcribed DNA copy of the viral genome is integrated into the host DNA. This integration requires interaction between two retroviral components: the viral enzyme integrase (IN) and specific sequences located at the termini of the linear viral DNA. A number of studies have shown that integration of viral DNA into the host DNA depends on at least two temporally and biochemically distinguishable activities of IN, processing and joining (for review, see refs. 1–3).

During processing, a site-specific endonuclease activity of IN removes 2 nt from each of the 3′-OH ends of the viral DNA, creating new CA-3′-OH ends and dinucleotides (4–7). In the joining reaction, the newly processed 3′-OH ends of the viral DNA are coordinately joined to 5′-phosphate ends produced by staggered cleavage of the host DNA by IN (6, 8–10).

In vitro assays for the processing and joining activities of IN have been described (4, 9, 10). The substrates are short duplex oligodeoxynucleotides homologous to either end of the viral DNA. The processing activity generates products analogous to those produced *in vivo*: an oligonucleotide duplex with a recessed CA-3′-OH end and a dinucleotide. This processed end can then be joined to other oligonucleotides in the reaction mixture, which presumably act as surrogates for host target DNA. In the most commonly used version of this assay, the 5′-phosphate end of the strand that will be processed is radiolabeled. Since this labeled strand is 2 nt shorter after processing, and a variable number of nucleotides longer after joining, products of both enzymatic steps can be separated by electrophoresis in denaturing polyacrylamide gels (4, 9, 10). Other versions of the processing assay, in which the 3′-OH ends of the strands to be cleaved are radiolabeled, have also been described (11–13).

Recently, we reported results from kinetic analyses of Rous sarcoma virus (RSV) IN which revealed that an oligomeric form of IN, minimally a dimer, was required for both processing and joining (14). Although the gel analyses used in those studies provided important information, they are extremely laborious and unsuited to routine kinetic studies. Here we describe a modified, rapid method to quantitate the processing activity of IN that does not depend on gel analysis. In this assay, the strand to be processed is radiolabeled near its 3′-OH end and PEI-cellulose is used to separate the unprocessed substrate from the labeled dinucleotide product of the reaction. The oligodeoxynucleotide joining assay has also been modified to make it more suitable for kinetic studies. Using these assay systems, we examined kinetic parameters of the reactions with wild-type RSV IN and several proteins mutated at single residues within a region believed to encode the catalytic domain of the IN enzymes.

MATERIALS AND METHODS

RSV IN. Nonfusion forms of wild-type and mutant RSV IN were produced and purified as described (15, 16). Purified proteins were stored at −70°C in 50 mM Hepes, pH 8.1/1% (vol/vol) thiodiglycol/0.5 M NaCl/40% (vol/vol) glycerol.

Oligodeoxynucleotide Substrates. Substrates were derivatives of duplex oligodeoxynucleotides that represent all or part of the last 18 bp of the RSV U3 region of the long terminal repeat. They were synthesized by standard solid-phase methods and purified by denaturing gel electrophoresis, and the concentrations were estimated from the OD_{260} by using calculated molar extinction coefficients. Annealing was done by mixing equimolar amounts of the complementary oligodeoxynucleotides in 20 mM Tris, pH 8.0/50 mM NaCl and heating for 10 min at 80°C, followed by cooling to room temperature over ≈4 hr.

To prepare a 3′-labeled duplex for the processing assay, the recessed end of an 18/17-mer oligodeoxynucleotide duplex lacking the 3′-terminal nucleotide of the (−)-strand was

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Abbreviations: HIV, human immunodeficiency virus; IN, integrase; RSV, Rous sarcoma virus.

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repaired by the addition of [α-^{32}P]dTTP (3000 Ci/mmol; ICN; 1 Ci = 37 GBq) with Sequenase (United States Biochemical). Free radioactive dTTP was removed by chromatography on DE-52 cellulose (Whatman). Unlabeled blunt duplex was added (final concentration, 100 μM), and the specific radioactivity of this substrate preparation (1000–3000 cpm/pmol) was determined by liquid scintillation counting.

To prepare substrates for the joining assay, the 5′-end of the U3 16(−)-strand oligodeoxynucleotide was labeled with ^{32}P (17). The radioactive strand was then annealed with a 2-fold excess of U3 18(+)-strand containing biotin on the 3′ end. Unlabeled biotinylated duplex was added to 50 μM.

Preparation of PEI-Cellulose Suspension. Eighty grams of PEI cellulose powder, fine mesh (Sigma), was suspended in 0.8 M LiCl/10 mM Tris, pH 7.3, to a total volume of 400 ml and incubated overnight at 4°C. Fine particles were then removed by several cycles of mixing, short centrifugation in a tabletop centrifuge (30 s, 400 × g), and subsequent exchange of the supernatant against the same volume of fresh buffer.

RESULTS

Assay for Processing Activity. The principles which underlie the simplified oligodeoxynucleotide processing and joining assays are illustrated in Fig. 1. The methods are applicable to analysis of any retroviral IN, using an oligodeoxynucleotide substrate whose sequence corresponds to the end of its unintegrated viral DNA. The particular examples shown in this report use RSV IN and an oligodeoxynucleotide duplex substrate that mimics the U3 terminus of RSV viral DNA (4, 14).

In the processing assay (Fig. 1*A*) the substrate is labeled with ^{32}P between the ultimate and the penultimate nucleotide at the 3′ end of the (−)-strand which is to be cleaved by IN. The amount of radiolabeled dinucleotide product is quantitated. To avoid the time-consuming steps of separating substrates from products by gel electrophoresis and quantitating by radioanalytic imaging, conditions were selected in which the oligodeoxynucleotide substrate but not the dinucleotide product binds efficiently to PEI-cellulose. Thus, batch adsorption onto PEI-cellulose and subsequent centrifugation allows rapid separation of the two. Under conditions described in Fig. 1 (legend), >99% of the full-length oligodeoxynucleotide is found in the pellet after centrifugation, whereas ≈40% of the dinucleotide product is found in the supernatant.

To determine the efficiency of separation according to size, a 5′-radiolabeled oligodeoxynucleotide was partially digested with DNase I. Components in the total digest and in the PEI supernatant were then compared in a denaturing polyacrylamide gel (Fig. 2, lanes 1 and 2). The predominant digestion products which remained in the PEI supernatant were 2 and 3 nt long. Traces of 4- and 5-nt products were also observed, but none of the full-length oligonucleotide was detected. Fig. 2 also shows results from an analysis of the products of a RSV-IN catalyzed processing reaction using a 3′-end-labeled substrate (lanes 3–5). Although predicted to be a dinucleotide, the major product migrated at a position approximately halfway between the 5- and 6-nt markers (lane 4), and it remained in the supernatant after PEI extraction (lane 5). Studies with human immunodeficiency virus type 1 (HIV-1) and Moloney murine leukemia virus IN (7, 12) have shown that nucleophilic attack by certain alcohols (e.g., glycerol) or

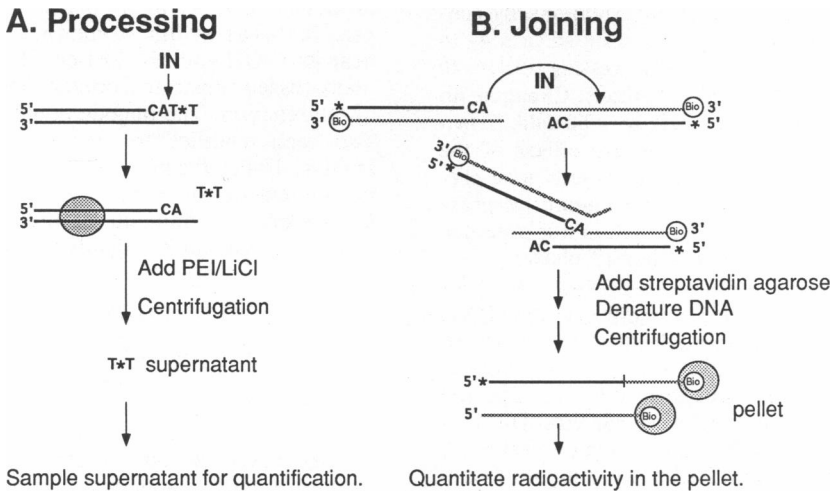

FIG. 1. Principles of the processing and joining assays. (*A*) Processing. Unless stated otherwise, reactions are carried out at 37°C in 50 mM Hepes, pH 8.0/2 mM 2-mercaptoethanol/0.01% bovine serum albumin (Boehringer Mannheim)/3 mM $MnCl_2$/4% (vol/vol) glycerol/50 mM NaCl/12.5 μM 3′ labeled oligonucleotide duplex/2 μM IN monomer (volume, 10–40 μl). At selected times 4- to 10-μl samples are removed and the reaction is stopped by the addition of 195 μl of 50 mM EDTA. To this mixture, 700 μl of PEI slurry is added, and the suspension is briefly vortexed and then agitated for 10 min at room temperature. The samples are then spun for 10 min at 14,000 rpm in an Eppendorf centrifuge, 250 μl of the supernatant is carefully removed, and the released radioactivity is quantitated by liquid scintillation counting. To obtain a correction factor for the percentage of dinucleotide present in this portion of the supernatant (≈25% under the conditions described), in each experiment two samples containing gel-purified ^{32}P-labeled pTpT are extracted in parallel. (*B*) Joining. The reaction mixtures are set up as described above except that a U3 oligonucleotide duplex carrying a 5′ ^{32}P label on the recessed (−)-strand as well as a biotin group on the 3′ end of the (+)-strand is used as a substrate (18). Aliquots (5 μl) are removed after selected incubation times, and the reaction is stopped by the addition of 5 μl of 100 mM EDTA and 120 μl of 20 mM Tris, pH 8.0/10 mM EDTA. Next, 70 μl of streptavidin-agarose suspension [50% (vol/vol) in 10 mM Tris, pH 8.0/1 mM EDTA] is added, followed by 1 hr of gentle agitation at room temperature. After centrifugation (5 min at 14,000 rpm in an Eppendorf centrifuge), the supernatant solution is carefully removed and discarded. The agarose beads are twice washed for 5 min in 900 μl of denaturing solution (30 mM NaOH/0.2 M NaCl/1 mM EDTA) and then given a final wash in 900 μl of 10 mM Tris, pH 8.0/1 mM EDTA. An oligodeoxynucleotide duplex carrying only the radioactive label is used as a control for nonspecific binding to the affinity material, whereas a duplex carrying both the biotin group and the radioactive label on the (+)-strand serves as a positive control for binding. The radioactivity bound to the beads is determined by liquid scintillation counting. If joining to the (−)-strand and (+)-strand of the target molecule is carried out with equal efficiency, the radioactive products bound to the immobilized streptavidin in this assay would represent 50% of the actual integration products. Circles labeled Bio symbolize biotin; stippled spheres symbolize PEI-cellulose (*A*) or streptavidin-agarose (*B*). Stars indicate ^{32}P radiolabel.

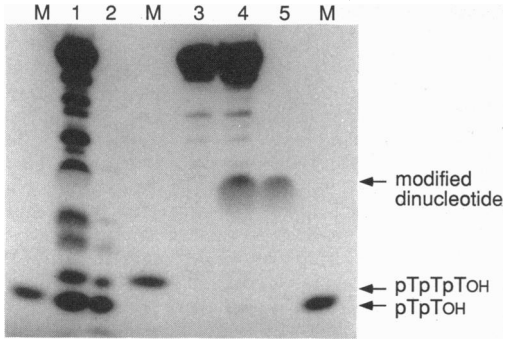

FIG. 2. Size fractionation of oligodeoxynucleotides by extraction with PEI-cellulose. Two-tenths of a picomole of 5′ ^{32}P-labeled oligonucleotide U3 18(−) was incubated with 0.5 μg of DNase I in 20 μl containing 50 mM Tris (pH 7.5), 10 mM $MgSO_4$, and 0.1 mM dithiothreitol. After 20 min at 30°C, 55 μl of 25 mM EDTA was added to stop the reaction. A 15-μl sample of this mixture was removed to be used for the analysis shown in lane 1. The remainder was extracted with PEI-cellulose as described in Fig. 1*A*. Equivalent samples before (lane 1) and after (lane 2) extraction with PEI were analyzed by denaturing PAGE (20% acrylamide, 7 M urea). An analogous extraction was performed with products of a RSV IN-catalyzed processing reaction using a 3′ ^{32}P-labeled oligodeoxynucleotide substrate. Lane 3, before incubation with IN; lane 4, after incubation with IN; lane 5, supernatant after PEI extraction of the material shown in lane 4; lanes M, markers for di- and trinucleotides (pTpT-OH and pTpTpT-OH), as indicated.

the 3′-OH group of the (−)-strand oligonucleotide, rather than by water, produces alcohol adducts and cyclic dinucleotides that migrate more slowly than the unmodified dinucleotide product. Under the standard conditions used in this study (3 mM $MnCl_2$, 4% glycerol), virtually all the dinucleotide produced by RSV IN is released in a modified form. Since mainly unmodified dinucleotides are produced from a dideoxy-terminated substrate that lacks a 3′-OH at the end of the (−)-strand (data not shown), we conclude that the slower migrating band produced in these reactions is almost exclusively a cyclic dinucleotide. However, cyclization does not interfere with the assay; the modified products of the processing reaction are separated from the substrate as efficiently as would be predicted for the unmodified product (Fig. 2, compare lanes 1 and 2 with lanes 4 and 5).

RSV IN produces a secondary, trinucleotide endonuclease cleavage product (4, 10) and other short oligomers, which are presumed to be by-products of the subsequent joining reaction. However, the contribution of such products to the PEI supernatant should be negligible at early times in the reaction, before joining is detected. Analysis of 5′-radiolabeled reaction products in sequencing gels shows that under our standard conditions (2 μM IN, 12.5 μM substrate) the rate of formation of the −2 product is linear for about 3 min. Furthermore, within this early phase, >80% of the product reflected specific cleavage at the −2 position, and during this time no products of the secondary cleavage at −3 (4, 10) or of the joining reaction were detectable (Fig. 3).

The effect of various reaction conditions on the early phase of processing *in vitro* was next examined. The rate was observed to increase from pH 6 to pH 9, with optimal activity at about pH 9 (Fig. 4*A*). Since alkaline pH promotes oxidation and precipitation of Mn^{2+} and also destabilizes the oligonucleotide duplexes, processing activity could not be assayed under more basic conditions and standard reactions were performed at pH 8.0. No processing activity was detected at $MnCl_2$ concentrations lower than 0.6 mM (Fig. 4*B*). The rate of processing increased with $MnCl_2$ concentration until it reached a plateau at ≈3 mM, and this was chosen for the standard assay. Since the RSV IN used in these studies requires glycerol and relatively high salt concentrations to

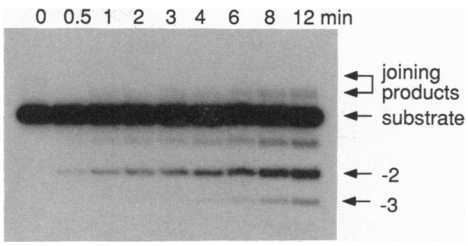

FIG. 3. Products of the processing reaction. In this experiment 20 μM 18-bp duplex labeled at the 5′ end of the strand to be processed was incubated with 2 μM (monomer) wild-type IN at 37°C in 40 μl of standard reaction buffer (Fig. 1*A*). At the indicated times, 4-μl samples were removed and analyzed by denaturing PAGE.

remain in solution, their effects on the assay were also determined. Varying the glycerol concentration between 1% and 18% had no significant effect on the rate of processing (data not shown). In contrast, increasing the ionic strength above the optimal concentration of ≈50 mM NaCl decreased the rate of the reaction (Fig. 4*C*). Under standard conditions, only 50% of the maximal activity was observed at 160 mM NaCl and no activity was detected at 0.5 M.

Processing Activity of Mutant IN Proteins. We have reported the preparation and analysis of a series of mutant RSV IN proteins with single amino acid substitutions in residues that are invariant or highly conserved in analogous proteins of all retroviruses and retrotransposons and in some bacterial transposases (16, 19). By comparing product band intensities in a gel-based assay it was found that such substitutions resulted in a more or less severe decrease in processing activity depending on the site and the nature of the mutation. The PEI method was used to obtain quantitative data on the early rate of processing by several of these mutant proteins. A time course of the formation of processed product by wild-type and mutant RSV IN proteins under standard conditions is shown in Fig. 5*A*. Wild-type IN was the most active (0.18 min^{-1}). Processing catalyzed by the mutant IN proteins tested proceeded at apparent rates 4–60 times lower (T66A and T66Q, 0.045 min^{-1}; F126A, 0.028 min^{-1}; S85C, 0.025 min^{-1}; K164A, 0.012 min^{-1}; S85G, 0.017 min^{-1}; D121E, 0.003 min^{-1}). Values for the two S85 mutants represent overestimates, since independent gel analyses revealed that ≈75% of the product produced by these mutants resulted from cleavage at the −3 position. Thus, the authentic processing product (−2) was generated at apparent rates about 4-fold lower than the values given above.

Rates determined for F126A, K164A, and S85G did not show a measurable increase over a range of substrate concentrations tested (Fig. 5*B*). This suggests that their defects reflect lower catalytic activities rather than decreases in the

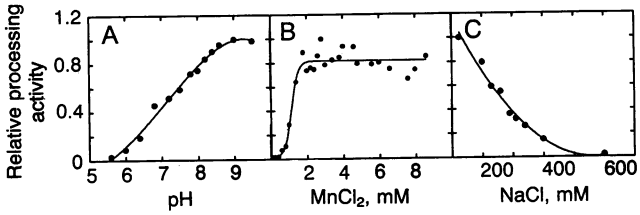

FIG. 4. Influence of reaction components on the processing activity of RSV IN. IN (2 μM monomer) was incubated with 12.5 μM oligodeoxynucleotide duplex in 20 μl at 37°C. Reactions were stopped after 2 min, and the amount of dinucleotide released was determined by PEI extraction. Reaction conditions were modified from the standard conditions given in Fig. 1*A* as follows: In *A*, the buffer was 50 mM Mes (pH 5.6–6.4), 50 mM Hepes (pH 6.8–8.2), or 50 mM Ches (pH 8.5–9.5). In *B* and *C* various concentrations of $MnCl_2$ or NaCl were present. All data points represent mean values from experiments done in duplicate or triplicate.

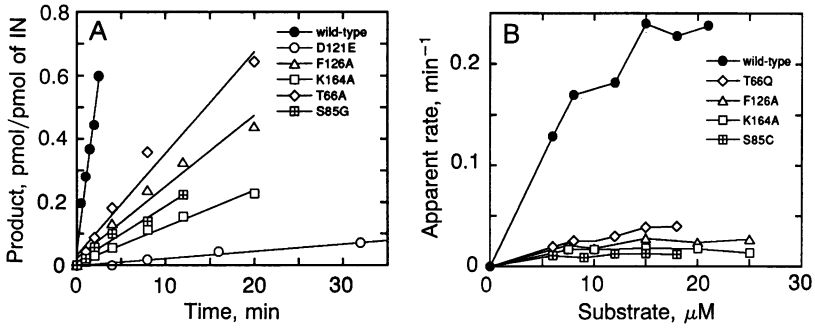

FIG. 5. Processing activity of wild-type and several mutant RSV IN proteins. (*A*) All proteins were incubated with 3′-^{32}P-labeled substrate under standard conditions (Fig. 1*A*). At the indicated times, samples were removed and analyzed by PEI extraction. Data points represent mean values from duplicate experiments. The data were fit to a linear equation to obtain the early rates (per IN monomer) cited in the text. (*B*) Same conditions as in *A*, except that substrate was varied from 8 to 25 μM. Replot of early rates determined versus substrate concentration is shown.

affinities for substrate. However, because the activities of most of these mutants are so low, variations of less than an order of magnitude might be undetectable in this assay. Thus, we cannot be certain that the highest substrate concentration which is practically feasible in the oligonucleotide assay represents saturating conditions, and processing activities of the mutant IN proteins are given as relative values determined under defined conditions.

Joining Activity of Mutant IN Proteins. A rapid solution assay for joining (Fig. 1*B*) was also employed to obtain more quantitative data on this activity of the retroviral IN proteins. The assay utilizes 5′-radiolabeled, biotin-complexed oligodeoxynucleotides (18), and the radioactive products of the joining reaction are collected by batch adsorption onto streptavidin-agarose beads. A "preprocessed" substrate [U3 18(+)/U3 16(−)] was used to follow the joining reaction independently from the processing reaction (9, 10). The results of such an experiment with wild-type IN and three of the mutant proteins are shown in Fig. 6. When the data obtained were fit to a linear equation, the relative effect of a given amino acid substitution on the rate in this reaction generally paralleled that on processing: wild type, 0.057 min^{-1}; T66A, 0.043 min^{-1}; K164A, 0.021 min^{-1}; D121E, 0.001 min^{-1}. However, closer analyses of these and other data sets revealed that the generation of joined product was not linear within the observed period of time. Instead, the linear phase of the reaction (≈0.04 min^{-1} for wild type) was preceded by what appears to be an exponential "burst" phase.

DISCUSSION

In this study we used rapid solution assays to facilitate biochemical analysis of both the processing and joining activities of retroviral IN proteins. One aim was to define conditions for obtaining relative rates of processing and of joining, so that effects of mutations or inhibitors on these two activities could be quantitated separately. When analyzing the processing reaction it is important to consider that both the oligodeoxynucleotide duplex substrates and the processed products can serve as substrates for the joining reaction, and it is not possible experimentally to separate these two steps. Most studies published to date have measured processing activity by incubating small amounts of substrate with a significant molar excess of protein; the amount of cleaved oligonucleotide present was determined after a single incubation time usually in the range of ≥1 hr (16, 19–22). For studies using substrate labeled at the 5′ end of the strand to be cleaved, the conversion of the radiolabeled processed product into joined products makes quantitation particularly difficult.

To obtain more accurate measurements of the processing activity alone, the oligodeoxynucleotide assay was modified in several ways. The reactions were run in an excess of substrate over enzyme and the released dinucleotide product, which does not serve as a substrate for further reactions, was quantified. The high sensitivity of the PEI extraction method makes it possible to measure processing activity after short incubation periods (<3 min for wild type) during which no joining is detectable. Furthermore, large numbers of samples can be analyzed within a relatively short period of time. Since important details concerning the mechanism of the overall reaction are still unknown, it is not possible to calculate actual rate constants for catalytic steps from these experiments. However, they provide a practical and useful approach for quantitating relative processing activities.

In the course of establishing optimal conditions for analysis of wild-type IN, we have found that glycerol at 1–18% has no significant effect on the reaction, but NaCl, which is needed to keep the protein in solution, is inhibitory. Processing activity increased from pH 6 to pH 9. A pH optimum of the processing reaction in the alkaline range has previously been reported for avian myeloblastosis virus (11) and HIV-1 (23) IN. Since this reaction involves a nucleophilic attack on the phosphodiester bond at the site of cleavage, this alkaline optimum may reflect the increasing deprotonation of the hydroxyl groups on the nucleophiles under more alkaline conditions.

Under our standard conditions, virtually all of the dinucleotide product of the RSV IN processing reaction was found to be in a cyclic form. This is somewhat different from what has been reported for HIV-1 IN, where both unmodified and modified forms of the dinucleotide products were observed in comparable amounts (7, 12), and for avian myeloblastosis virus, where only an unmodified product was observed (11). However, these analyses were performed under conditions significantly different from those described here. Furthermore, we have observed that changing reaction conditions (e.g., using Mg^{2+} instead of Mn^{2+}) can dramatically alter the ratio of unmodified to modified dinucleotide product generated. Therefore it seems unlikely that the difference between our observations and the results reported previously

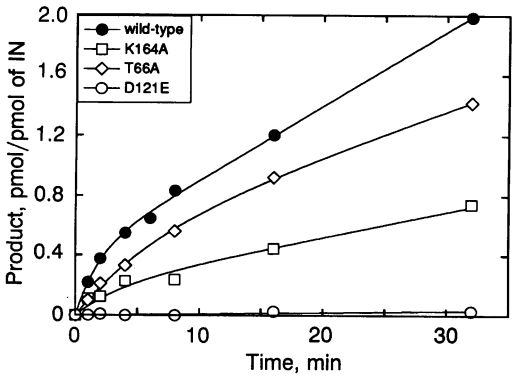

FIG. 6. Joining activity of wild-type and mutant IN. IN proteins (2 μM) were incubated with a 20 μM mixture of 5′ ^{32}P-labeled and 3′-biotinylated duplex substrate molecules as described in Fig. 1*B*. The data were fit to an equation describing an exponential burst followed by a linear phase ($y = Ae^{(-kt)} + mt$) using the commercially available software KALEIDOGRAPH (Synergy Software, Reading, PA).

reflects a fundamental difference in mechanisms employed by these proteins.

Since processing is required for integration, substances which interfere with this activity of human retroviral IN would represent potential candidates for antiviral therapy. Compounds which inhibit the processing and joining reactions of HIV-1 IN have recently been identified by sequencing-gel analysis (24). In our hands, the PEI assay is a far more efficient method to measure the ability of compounds to inhibit processing (G.W.M. and A.M.S., unpublished observations) and could prove useful for large-scale screening.

The PEI processing assay was used to quantitate the effects of single amino acid changes in residues highly conserved among IN proteins. We observed that, except for T66Q, initial rates with the mutant RSV IN proteins do not show a detectable increase with increasing substrate concentration (Fig. 5*B*). The simplest explanation of these data is that the enzyme is saturated with substrate and that the decreases in processing reflect decreased rates of the catalysis rather than lower substrate affinities. This is consistent with the fact that all of the proteins tested have mutations in invariant or highly conserved residues in what appears to be an evolutionarily conserved catalytic domain of the enzyme (ref. 25; J. Kulkosky, R. A. Katz, G.W.M., and A.M.S., unpublished work). However, because of the low activity of these mutant proteins and practical limitations on the amount of oligodeoxynucleotide substrate that can be used in these reactions, it is not possible to completely rule out effects on substrate binding.

Quantitation of the level of joining using the described modifications was also more rapid and reproducible than that obtained by sequencing-gel evaluation. Results with both wild-type and mutant RSV IN proteins revealed that an exponential "burst" in production of joined product was followed by a linear phase (Fig. 6). Our current interpretation of this observation is that the rate-limiting step for this reaction is the release of product. In this case, the fast phase would represent the joining of substrate molecules initially bound to IN and accumulation of product at the active site of the enzyme. The slower rate in the linear phase would then reflect the release of joined products. Although relatively high concentrations of enzyme and substrate were used, the amplitude of the "burst" is much lower than one molecule per enzyme monomer. This could be explained by inactive IN molecules in the preparation, by a requirement for higher-order IN multimers to generate joined products, or by a low-affinity (K_d in the 10-μm range) of IN for the substrate analogs. Another possibility is that a significant proportion of the enzyme–substrate complexes formed are incorrect, or "unproductive." Since important parameters such as the affinity of IN for the substrate analogs and the stoichiometry of the IN–substrate complex have not been determined, none of these possibilities can be excluded at this time.

Using this kinetic approach in analysis of processing by mutant IN proteins, we obtained results that agreed generally with qualitative estimates reported previously (16, 19–22). The conservative substitution of D121E, one of the three invariant residues that define the D,D(35)E region (16), produced the most dramatic decrease in activity. The S85 mutations were next in the order of defectiveness. The substitution of three other conserved residues (T66, F126, and K64) generated proteins with higher activity, but the rates determined for these proteins are still significantly reduced from wild-type levels. Comparison of our rate determinations with estimates obtained in other studies of the same enzymes reveals that previous reports most often overestimated the level of activity, especially of mutants with moderate defects.

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